Graphene-polymer-enzyme hybrid nanomaterials for biosensors

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(54) **Title**: GRAPHENE-POLYMER-ENZYME HYBRID NANOMATERIALS FOR BIOSENSORS

(57) **Abstract**: The invention relates to a general chemical method for the synthesis of biocompatible hybrid nanomaterials which can be used in the development of new- type enzyme based biosensors. A one-step facile method is presented, in which polyethylen - imine (PEI) serves as both a reducing agent for the reduction of graphene oxide (GO) into reduced graphene oxide (RGO) and a bio - logical matrix for accommodation of enzymes.

Graphene-Polymer-Enzyme Hybrid Nanomaterials for Biosensors

The invention relates to a general chemical method for the synthesis of biocompatible hybrid nanomaterials which can be used in the development of new-type enzyme based biosensors.

Background

Complete chemical exfoliation of graphite flakes can generate single-layered and water soluble individual graphene oxide (GO) sheets. However, GO is electrically insulating, and if the material is to be used for electronic applications or as electrode materials, the conductivity of the material thus needs to be restored. This can be achieved via chemical or thermal reduction of GO into its reduced form (RGO). Several reducing agents, including hydrazine (N_2H_4),1-dimethylhydrazine,3-sodium borohydride, and hydrogen quinine, have been attempted for reducing GO, showing results with variant efficiency.

Among the above reducing agents, hydrazine is arguably the mostly common used agent. However, hydrazine is not an environmentally and biologically friendly agent. In addition, RGO suspensions prepared by hydrazine have a limited stability ranging from a few hours to days, depending on experimental conditions. CN102850795 discloses an example of a method where hydrazine is used as the reducing agent for reducing GO to RGO. After the reduction of GO to RGO by hydrazine, RGO is mixed with a Ferrocene grafted PEI thereby forming non-covalent bonds between PEI and RGO.

Shanli Yang *et al*. (Microchim Acta 2013, **180**, page 127-135) discloses an alternative method for reducing GO to RGO, wherein a biosensor containing a GO solution is reduced to RGO by immersing a glass carbon electrode into the solution.

Thus, the development of a general chemical route towards the preparation of electrically conductive and biocompatible RGO is of particularly desired for facilitating the use of RGO in biological environments.

Description of the invention

The invention discloses a one-step green reduction and polymeric derivation of graphene oxide (GO), which makes it possible to produce stable and biocompatible reduced GO (RGO) nanosheets. More specifically, here is disclosed a facile environmentally friendly and practical approach to one-step green reduction of graphene oxide by the polymer polyethylenimine (PEI) in an aqueous medium with the simultaneous formation of covalent linkage between the polymer and the RGO.

Disclosed herein is therefore a method for preparing hybrid biofunctional composites comprising reduced graphene oxide (RGO), polyethylenimine (PEI) and an enzyme. The method comprising the steps of A) providing an aqueous solution of graphene oxide (GO); B) reducing the GO by adding PEI to the aqueous solution of GO thereby obtaining an aqueous RGO-PEI solution, and C) mixing the aqueous RGO-PEI solution with an enzyme thereby obtaining the hybrid biofunctional composites.

The RGO and the PEI form covalent bonds and PEI forms a biocompatible matrix electrostatically encapsulating the enzyme inside the matrix. Thus, the enzyme binds non-covalently to the RGO supported PEI.

By the above method is obtained a hybrid biofunctional composite comprising a combination of a conducting element, i.e. RGO, polymer cage/matrix defined by the PEI polymer covalently bound to RGO, and a biological catalyst in the form of an enzyme. The non-covalent bonding between the enzyme and the remaining part of the hybrid biofunctional composites, i.e. RGO-PEI, ensures that the enzyme is not altered when contained inside the PEI matrices, as the enzyme does not form any bonds with the PEI matrices material or the RGO. Instead, the enzyme is allowed to move ‘freely’ inside the polymer cage to the extent that is defined by the size of the enzyme and the polymer matrix pockets.

The enzyme well retains its structures and catalytic activity because it is located in a biological compatible matrix or environment. In other words, the catalytic properties of the enzyme is maintained even though the enzyme is coupled to RGO and PEI.

The polymer PEI is further environmentally compatible and environmentally friendly, making it an environmentally better alternative than hydrazine. It is able to form covalent bonds with GO at the same time reducing GO to RGO, the latter which is an excellent conducting material. Thus, using PEI as the reducing agent, environmentally and biologically unfriendly agents like e.g. hydrazine, can be avoided.

In addition, RGO suspensions prepared by hydrazine have a limited stability ranging from a few hours to days, depending on experimental conditions. This instability problem is avoided when the reduction of GO to RGO is obtained using PEI.

Thus, by the above method is obtained a general chemical route for the preparation of electrically conductive and biocompatible RGO, which can be used in biological environments.

Also disclosed herein is a hybrid biofunctional composite comprising reduced graphene oxide (RGO), polyethylenimine (PEI) and an enzyme, wherein the RGO and PEI form covalent bonds and wherein PEI forms a biocompatible matrix electrostatically encapsulating the enzyme inside the matrix. Thereby the enzyme 'binds' non-covalently to the RGO-supported PEI.

Also disclosed herein is a hybrid biofunctional composite according to the above and prepared by the method disclosed herein.

Herein is further disclosed an electrode-composite structure comprising hybrid biofunctional composites as described above.

Brief description of the drawings

Figure 1 discloses a method for preparing RGO-PEI composites.

Figure 2a shows a cuvette (left-hand side) with a solution of the as-prepared GO nanosheets and the corresponding UV spectrum of the solution (right-hand side).

Figure 2b shows a cuvette (left-hand side) with a solution of RGO-PEI nanosheets and the corresponding UV spectrum of the solution (right-hand side).

Figure 2c shows a cuvette (left-hand side) with a solution of RGO nanosheets reduced by hydrazine (N_2H_4) and the corresponding UV spectrum of the solution (right-hand side).

Figures 3a-3d show AFM images of GO (figure 3a), RGO reduced by N_2H_4 (figure 3b), RGO-PEI (figure 3c), and RGO-PEI/GOx composite (figure 3d) on mica, where GOx is glucose oxidase.

Figures 4a-4b show high-resolution AFM images of RGO-PEI structures on mica with different resolutions.

Figures 5a-5d show XPS spectra of RGO-PEI.

Figure 6 is a table summarizing the elemental analysis of the amount for surface oxygen groups in GO, RGO-N_2H_4 and RGO-PEI by XPS.

Figure 7a shows the FTIR spectra of GO, RGO-N_2H_4 ('marked RGO') and RGO-PEI.

Figure 7b is a table listing the peak modes (given in cm^{-1}) observed for GO and RGO-PEI in figure 7a.

Figure 8 is a schematic overview of the preparation of hybrid biofunctional composites.

Figure 9a shows the UV-Vis absorption spectra of GOx before and after conjugation to RGO-PEI.

Figure 9b shows the mass ratio of absorption of the GOx to RGO-PEI obtained from the data in figure 9a.

Figure 9c is a table summarizing the results shown in figures 9a and 9b.

Figure 10a shows the UV-Vis absorption spectra of ChOx before and after the adsorption to RGO-PEI.

Figure 10b shows the mass ratio of the absorption of ChOx to RGO-PEI shown in the UV-VIS spectra in figure 10a.

Figure 10c is a list of the data analysis of the mass ratio of ChOx to RGO-PEI based on the results shown in figures 10a and 10b.

Figure 11a shows the electrocatalytic oxidation of cholesterol with different concentrations at a glassy carbon electrode (GCE) surface coated with the RGO-PEI-ChOx composite.

Figure 11b shows a calibration plot of ChOx biosensors in response to cholesterol based on the data shown in Figure 11a.

Figure 12a shows the electrocatalytic oxidation of glucose at edge plane graphite electrode (EPG)/RGO-PEI/GOx.

Figure 12b shows the amperometric response of EPG/RGO-PEI/GOx with successive addition of glucose in 10 mM PBS (pH 7.0) containing 0.8 mM Fc-COOH at 0.35 V.

Figure 12c shows the amperometric responses versus the glucose concentration.

Figure 12d shows a calibration plot of the biosensor for glucose based on the results shown in figure 12a.

Figure 13a-b show measurements of the glucose levels in human blood samples using the hybrid biofunctional composite sensor according to the invention.

Figure 14a-b show measurements of the concentration of glucose in human blood sample obtained from Glostrup Hospital, Denmark using the hybrid biofunctional composite sensor according to the invention.

Figure 15 shows the long term stability of the hybrid biofunctional composites sensor according to this invention.

Description of preferred embodiments

Disclosed herein is therefore a general chemical method for preparing hybrid biofunctional composites comprising reduced graphene oxide (RGO), polyethylenimine (PEI) and an enzyme.

In one or more embodiments the enzyme has an isoelectric point below 10.

In one or more embodiments the enzyme is chosen from the group of glucose oxidase (GOx), cholesterol oxidase (ChOx), horseradish peroxidase (HRP), alcohol dehydrogenases (ADH), and Choline oxidase.

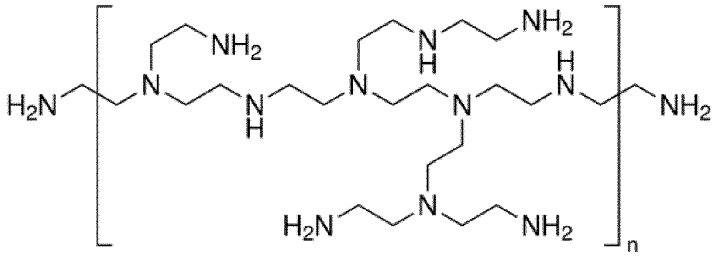
The method for preparing hybrid biofunctional composites comprising the steps of providing an aqueous solution of graphene oxide (GO), reducing the GO by adding PEI to the aqueous solution of GO thereby obtaining the aqueous RGO-PEI solution, and mixing the aqueous RGO-PEI solution with an enzyme thereby obtaining an hybrid biofunctional composite.

The RGO and the PEI form covalent bonds and PEI forms a biocompatible matrix electrostatically encapsulating the enzyme inside the matrix. More specifically, PEI normally forms a matrix positioned on the plane and edges of the RGO nanosheets, where the PEI matrix forms cages which electrostatically encapsulates the enzyme inside the matrix. Thus, the enzyme binds non-covalently to the RGO supported PEI.

In one or more embodiments, the PEI polymer has an average polymeric length of at least 60.000 monomeric units. Alternatively, the PEI polymer has an average

polymeric length of at least 10.000 monomeric units or at least 25.000 monomeric units.

In one or more embodiments, the PEI polymer has monomeric units with the molecular formula



In one or more embodiments the obtained solution after PEI is added is stirred for between 30 min. – 90 min., or 45 min. – 75 min., or for 60 min. at a temperature between 70-120 ˚C, or between 80-110 ˚C, or between 90-100 ˚C, or at 95 ˚C.

In one or more embodiments, mixing the aqueous RGO-PEI solution with the enzyme in step is done at a temperature between 1-10˚C, or between 2-8˚C, or between 3-6˚C, or between 4-5˚C, or at 4˚C for between 6-24 hours, or between 8-18 hours, or between 10-14 hours.

In one or more embodiments the mixture obtained when mixing the aqueous RGO-PEI solution with the enzyme is centrifuged at 8000 rpm for 15 minutes after being mixed at the temperature between 1-10˚C, or between 2-8˚C, or between 3-6˚C, or between 4-5˚C, or at 4˚C for between 6-24 hours, or between 8-18 hours, or between 10-14 hours.

In one or more embodiments the method for preparing hybrid biofunctional composites further comprising the steps of washing the solution obtained when mixing the aqueous RGO-PEI solution with the enzyme with phosphate buffered saline (PBS), and successively centrifugating the solution, e.g. three times, to remove loosely bound enzymes.

The hybrid biofunctional composite produced by the above described method may in one or more embodiments be used as an enzyme-based biosensing material for a graphene based biosensors.

Alternatively, the hybrid biofunctional composite may be used for:

- conjugating toxic heavy metal ions such as Pb^{2+}, Hg^{2+} and Cd^{2+};
- clean environmental and water technology; or
- drug delivery where RGO-PEI captures and releases specific drugs.

The RGO is obtained using polyethylenimine (PEI) as both reducing agent and functional linker. PEI is a polymer with abundant amine groups, composed of ethylenimine moieties as the repeating unit. PEI is known as a highly branched, positively charged and water soluble polymer. In the past few years, PEI has received tremendous attention as versatile building blocks for the construction of adsorbents as a result of its high amine density and accessible primary amine sites on its branched chains.

The RGO-PEI material exhibited significant improvement of the biocompatibility, which could provide a microenvironment for the accommodation of different kinds of enzymes. Therefore, the biocompatibility and the excellent electron transfer properties of this RGO-PEI-enzyme hybrid material pave the way for its use in biosensing.

Similarly, GO contains oxygen functional groups on their basal planes and edges. Therefore, GO could show high affinity to amines or amine containing molecules. When PEI is attached to GO nanosheets the residual amine groups in PEI can exhibit good adsorption capacity for anionic materials, such as polyanions and negatively charged organic, inorganic and biological molecules .

Wet-chemical synthesis of RGO based on PEI reduction is illustrated schematically in figure 1. A typical procedure for preparing RGO-PEI composite is conducted by mixing 400 μl 0.1 g/ml PEI with 80 ml H_2O, and then adding 20 ml 0.1 mg/ml GO. The mixed solution is normally stirred at 95° for 1 h. The change of color from brown to black indicates the reduction of GO to RGO by PEI .

The Graphene oxide (GO) is normally prepared by the modified Hummer's method with graphite flake <20 μm, used as a starting material. Preparation of graphene oxide (GO) involves a two-step process, where pre-oxidized graphite is prepared in a first step. Graphite powder (5.0 g) is slowly added into concentrated H_2SO_4 solution (7.5 ml) containing P_2O_5 (2.5 g) and $K_2S_2O_8$ (2.5 g) kept in a hot water bath (80°C) under strong stirring for 3 h. After cooling to the room temperature and diluting with Milli-Q water, the dark green mixture is filtered and washed several times until waste solution pH reaching neutral. The pre-oxidized graphite powder is afterwards collected and dried in air at room temperature overnight.

In the second step, pre-oxidized graphite powder (1.0 g) is slowly added to a concentrated H_2SO_4 solution (23 ml) in an ice-water bath (0 °C). $KMnO_4$ (3.0 g) is then added to the mixture under slow stirring keeping the whole process below 20 °C. After removing the ice-water bath, the mixture is reacted at 35 °C for 2 h with stirring and Milli-Q water (46 ml) added. After a few minutes, Milli-Q water (137.5 ml) and 2.5 ml of a 30% H_2O_2 solution are further added to the mixture, leading to the solution colour rapidly changing to bright yellow. The mixture is then washed with a 1:10 HCl solution (v/v, 250 ml) and filtered to remove residual metal ions. The raw GO suspended in Milli-Q water is centrifuged at a high rotation speed (12000 rpm min^{-1}). The supernatant containing highly dispersed and stable GO nanosheets is afterwards collected. To remove residual salts and acids, the supernatant is further dialyzed using a dialysis tube (with a cut-off molecular weight of 12000-14000) for at least one week by changing water bath regularly (2-3 times per day).

As mentioned above, during the synthesis of the RGO-PEI shown in figure 1, the colour of the dispersion changed gradually from brown to black over a period of approximately 60 min. Figure 2a shows a picture (left-hand side) of a cuvette with a solution of the as-prepared GO nanosheets and the corresponding UV spectrum of the solution (right-hand side). The UV spectrum of GO shows two absorption bands at 232 nm (marked as 102) and 302 nm (marked as 104), which are typical for GO.

Figure 2b shows a picture (left-hand side) of a cuvette with a solution of RGO-PEI nanosheets and the corresponding UV spectrum of the solution (right-hand side).

The UV spectrum of RGO-PEI shows one absorption band at 260 nm (marked as 106).

For comparison, figure 2c shows a picture (left-hand side) of a cuvette with a solution of RGO nanosheets reduced by hydrazine (N_2H_4) and the corresponding UV spectrum of the solution (right-hand side). The UV spectrum of RGO-hydrazine shows one absorption band at 266 nm (marked as 108) being close to the absorption band observed in figure 2b for RGO-PEI.

The RGO-PEI composites are analysed systematically by atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS), Fourier transform infrared (FTIR) spectroscopy, Raman spectroscopy, and thermo gravimetric analysis (TGA).

Considering the excellent dispersibility in water for the obtained RGO-PEI nanosheets, their single-sheet nature can be studied using AFM. The cross-sectional view of AFM images are shown for GO in figure 3a, RGO reduced by N_2H_4 in figure 3b, RGO-PEI in figure 3c, and RGO-PEI/GOx composite in figure 3d, where GOx is the enzyme glucose oxidase.

The dimensions in figures 3a-3d are 5 × 5 μm^2 in figure 3a and figure 3b, 2.5 × 2.5 μm^2 in figure 3c, and 1.8 × 1.8 μm^2 in figure 3d. The height of GO and RGO in figure 3a and figure 3b are about 0.8 nm. The height of RGO-PEI nanosheets in figure 3c is 2.1 ~ 2.5 nm, and the height of RGO-PEI/GOx composite in figure 3d is about 7.6 ~ 8.3 nm.

The average thickness of a single GO sheet is found to be 0.8 nm, the average thickness of the RGO reduced by hydrazine to be 0.9 nm, and the average thickness of the RGO-PEI between 2.1 nm ~ 2.5 nm. The increase in average thickness of RGO-PEI compared to RGO reduced with hydrazine could be attributed to the capping reagents PEI on their surface to replace the oxygen-containing functional groups after the reduction and covalent linkage.

Figure 4 shows cross-sectional views of AFM images of RGO-PEI structures on mica with a resolution of 5 × 5 μm^2 in figure 4a, and 1 × 1 μm^2 in figure 4b.

The XPS spectra of RGO-PEI is shown in figures 5a-5d, where figure 5a shows the XPS spectrum up to 1200 eV, figure 5b is a close-up of the ~285 eV peak representing the C1s, figure 5c is a close-up of the ~400 eV peak representing the N1s, and figure 5d is a close-up of the ~532 eV peak representing the O1s.

As shown in the table in figure 6, the surface oxygen groups in GO are about 30.2 %. The percentage of oxygen decreases to 11.64 % for RGO-N_2H_4 and similarly to 14.44 % for RGO-PEI. Thus, the C/O ratio in the RGO-PEI is increased remarkably after the reduction reaction to a level similar to that observed in RGO prepared by hydrazine.

The appearance of an N peak for RGO-PEI compared to that of GO and RGO-N_2H_4 indicates the attachment of PEI onto the RGO. The N1s XPS spectra of RGO-PEI shown in figure 5c suggest the presence of amide (399.1 eV) and amine (400.2 eV). The O1s core-level spectrum shown in figure 5d can be fitted to two peaks at 531.5 eV and 532.7 eV, as is expected.

Figure 7a shows the FTIR spectra of GO, RGO-N_2H_4 (marked RGO) and RGO-PEI and figure 7b is a table listing the peak modes (given in cm^{-1}) observed for GO and RGO-PEI. The O-H stretching mode and O-H bending mode are observed at 3415 cm^{-1} and 1386 cm^{-1}, respectively, both in GO and RGO-PEI. The O-H modes are observed as a relatively strong peak in pure GO but become significantly weaker in RGO-PEI composites.

The C=O stretching in the carboxyl acids and carbonyl groups is observed at 1725 cm^{-1} in GO, whereas it is observed at 1647 cm^{-1} for the N-C=O group in RGO-PEI.

As a comparison, the spectrum of RGO-N_2H_4 shows a more or less flat line with a weak peak in the fingerprint region at 1047 cm^{-1} representative of C-O stretching in the epoxy group. The same peak is observed in RGO-PEI as a weak peak and in

GO as a strong peak. The 878 cm^{-1} peak in GO is also attributed to the C-O group in the epoxy. This peak is not visible in the RGO-PEI and the RGO-N_2H_4 spectra. Skeletal vibration of graphitic domains are observed only in the GO at 1630 cm^{-1}. In RGO-PEI a weak C-N stretching is observed at 1450 cm^{-1}.

The structural characterization discussed above in connection with the preceding figures overall shows that PEI is covalently linked to the RGO nanosheets to form a biocompatible matrix. The PEI matrix therefore provides biocompatible microenvironments for accommodation of enzymes through electrostatic encapsulation. The RGO-PEI-enzyme nanocomposites can be cast into thin films on electrode surfaces, whereby the enzymes retains their catalytic activity. Thus, the resulting RGO-PEI materials described above provide a large electrochemically active surface for the adsorption of high amount of enzymes which can be used for highly sensitive and selective biosensors.

Figure 8 is a schematic overview of the preparation of hybrid biofunctional composites comprising reduced graphene oxide (RGO), polyethylenimine (PEI) and an enzyme from here referred to as the RGO-PEI-enzyme hybrid material. In the first step in the top part, the initial synthesis of the graphene oxide sheets 802 starting from graphite 801 is illustrated. This process may be performed using the modified Hummer's method as described previously in connection with figure 1.

In the next step shown lower part of figure 8, graphene oxide is simultaneously reduced and functionalized by the polymer PEI 803 to obtain RGO-PEI 805 as also described in connection with figure 1. The reduction / functionalization is followed by a loading of the enzyme 806 over the RGO-PEI matrix 805 to obtain the RGO-PEI-enzyme hybrid material 807.

The RGO-PEI-enzyme hybrid composites 807 may be obtained by mixing 800 µl 0.05 mg/ml RGO-PEI with 200 µl 1 mg/ml enzyme at 4˚C overnight thereby forming RGO-PEI-enzyme hybrid composites. The mixture is afterwards centrifuged at 8000 rpm for 15 minutes and the supernatant of the solution is collected for the determination of enzyme loading capacity over the RGO-PEI matrix.

The precipitate is collected and is normally washed with phosphate buffered saline (PBS) and successively centrifuged three times to remove loosely bound enzymes from RGO-PEI matrix. The immobilization efficiency of different enzymes may be determined indirectly by the UV absorption spectra by measuring the absorption of the free amount of enzyme in the supernatant and absorption of the actual amount of enzyme added before.

As illustrated in figure 8, PEI forms matrix-like cages/matrices 804 on the planes and at the edges of the GO sheets in the RGO-PEI material. When adding the enzyme 806, these PEI cages 804 facilitate an accommodation for the enzyme 806 such that the enzyme 806 is encapsulated electrostatically inside the PEI-formed matrix 804. Thereby the enzyme 806 does not form any covalent bonds to either the RGO material 802 or the PEI polymer 803 in the PEI matrices 804.

Figures 9-12 show further experimental data of two representative examples of enzymes accommodated in the RGO-PEI matrix; glucose oxidase (GOx) and cholesterol oxidase (ChOx) enzyme. Glucose and cholesterol are two crucial constituents of all human cells, and determination of glucose and cholesterol levels in blood is a crucial step for controlling and early diagnosis of many life threatening diseases such as diabetes and obesity.

Figure 9a shows the UV-Vis absorption spectra of GOx before and after the adsorption to the RGO-PEI matrix. Lines 901 and 902 show that the UV-Vis absorption spectra of pure GOx before (line 901) and after (line 902) centrifugation in the absence of RGO-PEI. As seen in the figure, it is apparent that these lines more or less are completely overlapping with one another, indicating that centrifugation alone does not decrease the enzyme amount in the solution and thus this is a good control experiment. Lines 903, 904 and 905 show the UV-Vis absorption spectra of GOx after centrifugation with RGO-PEI/GOx measured for three independent samples with good reproducibility as indicated by these lines overlapping each other.

Figure 9b shows the mass ratio of the absorption of GOx to RGO-PEI/GOx solutions shown in the UV-VIS spectra in figure 9a after centrifugation and compared to the

original GOx solution, where A and A_0 are the absorption of the solutions at 277 nm. As can be seen, the first two samples 901, 902 representing GOx shows a 1:1 ratio between the absorption before and after centrifugation of GOx, whereas a drop to 60% of the absorption compared to GOx is observed for the RGO-PEI/GOx samples 903, 904 and 905.

Figure 9c is a table summarizing the results shown in figures 9a and 9b. The high loading of enzyme GOx with a mass ratio of about 2 is achieved.

Figure 10a shows the UV-Vis absorption spectra of ChOx in line 1001, the RGO-PEI/ChOx spectrum after centrifugation in line 1002, and the RGO-PEI/ChOx spectrum and 12 hour after centrifugation in lines 1003 and 1004.

Figure 10b shows the ratio of absorption of the ChOx and RGO-PEI/ChOx solutions shown in the UV-VIS spectra in figure 10a after centrifugation and compared to the original ChOx solution, where A and A_0 are the absorption of the solutions at 277 nm. As can be seen, the first two samples 1001, 1002 representing ChOx shows a 1:1 ratio between the absorption before and after centrifugation of ChOx, whereas a drop to 60% of the absorption compared to ChOx is observed for the RGO-PEI/ChOx samples 1003 and 1004.

Figure 10c list of the data analysis of the mass ratio of ChOx to RGO-PEI based on the results shown in figures 10a and 10b. Similar to GOx, for ChOx a high loading with the mass ratio of about 2 is accomplished as well.

Figure 11a shows the electrocatalytic oxidation of cholesterol at a glassy carbon electrode (GCE) surface modified with RGO (GCE/RGO/ChOx) in 10 mM PBS (pH 7.0) in the presence of 0.8 mM ferrocenecarboxylic acid (Fc-COOH) as an electrochemical mediator. A scan rate of 50 mV/s is used in all the experiments, where the concentration of cholesterol is varied from 0 mM to 7 mM as shown in figure 11a.

Figure 11b shows a calibration plot (the line) of the biosensor for cholesterol based on the results shown in figure 11a (squares).

Figure 12a shows the electrocatalytic oxidation of glucose at edge plane graphite electrode (EPG)/RGO-PEI/GOx in 10 mM PBS (pH 7.0) in the presence of 0.8 mM Fc-COOH as an electrochemical mediator. A scan rate of 10 mV/s is used in all the experiments, where the concentration of glucose is varied from 0 mM to 7 mM as shown in figure 12a.

Figure 12b shows the typical amperometric response of EPG/RGO-PEI/GOx with successive addition of glucose in 10 mM PBS (pH 7.0) containing 0.8 mM Fc-COOH at 0.35 V. The amperometric responses versus the glucose concentration is shown in figure 12c and the calibration plot of the biosensor for glucose based on the results shown in figure 12a (squares) are shown as the line in figure 12d.

Figure 13a shows measurements of the glucose levels in human blood samples using the hybrid biofunctional composite sensor according to the invention. The round dots corresponds to datapoints obtained from measuring the different concentrations of standard glucose solutions and the blood drops corresponds to the datapoints obtained from measuring the glucose level of the two different blood samples by using the hybrid biofunctional composite sensor.

In figure 13b, a table is shown a comparison of the blood glucose detection method using the hybrid biofunctional composites sensor of this invention and a commercially available blood glucose monitoring device.

Figure 14a shows measurements of the concentration of glucose in human blood sample obtained from Glostrup hospital, Denmark by using the hybrid biofunctional composite sensor according to the invention. The round dots corresponds to datapoint obtained from measuring the different concentrations of standard glucose solutions and the blood drops corresponds to the datapoint obtained from measuring the glucose level of eleven different blood samples (supplied by Glostrup hospital, Denmark) by using the hybrid biofunctional composite sensor.

In figure 14b, a table is shown a comparison of the blood glucose detection method using the hybrid biofunctional composites sensor of this invention and a commercially available blood glucose monitoring device.

Figure 15 shows the long term stability of the hybrid biofunctional composites sensor of this invention for 30 days at 35°C. Amperometric measurements were performed with the biosensor for a period of 30 days during which the sensors were stored at 35°C. These conditions mimics the practical Summer conditions in some countries such as India and some south parts of China. The graph in figure 15 demonstrates that the stability is high even at very warm and humid weather conditions.

Chemicals and materials.

Graphite flakes (<20 μm, synthetic), D-(+)-glucose (≥ 99%), and glucose oxidase (GOx, from Aspergillus niger, 100,000-250,000 units/g solid) were purchased from Sigma-Aldrich. Ferrocenecarboxylic acid (≥97.0% (Fe)), poly(ethylenimine) solution (50% (w/v) in water, M_w = 750,000), K_2HPO_4 and KH_2PO_4 were purchased from Fluka. Phosphate buffer solutions (PBS) were employed as supporting electrolyte and the pH value was adjusted to 7.0 with K_2HPO_4 and KH_2PO_4. All chemicals were used as received. All solutions were prepared with Milli-Q water (18.2 MΩ).

Instruments

The UV-vis spectra were recorded using a single-beam spectrophotometer (HP8453, Hewlett Packard).

Atomic force microscopy (AFM) imaging was performed in the tapping mode using a 5500AFM system (Agilent Technologies, Chandler, USA).

X-ray photoelectron spectroscopy (XPS) analysis was carried out by an ESCALABMKII X-ray photoelectron spectrometer.

Fourier transform infrared spectra (FTIR) were recorded in the solid state using KBr substrates containing the target materials by a Perkin Elmer Spectrum.

Thermo gravimetric analysis (TGA, Netzsch STA 409PC) was reported in an N_2 atmosphere at a heating rate of 5 °C min^{-1}. A CHI 760C (USA) and an Autolab (Eco Chemie, The Netherlands) instrument in combination with a three-electrode system were used for electrochemical experiments. An edge plane graphite (EPG, d = 5 mm), a bright Pt wire and a saturated calomel electrode (SCE) were used as working electrode, counter electrode, and reference electrode, respectively. Electrolyte solutions were deoxygenated for 30 mins by Ar purified by Chrompack (oxygen $<$50 ppb). All systems were blanketed with an Ar-atmosphere during measurements.

The EPG was freshly cleaned by polishing on 1.0 μm, 0.3 μm, 0.05 μm Al_2O_3 slurry (Electron Microscopy Science, PA, USA) followed by ultra-sonication in Millipore water. Then the RGO-PEI-enzyme hybrid material was drop casted on the electrode surface for further electrochemical characterization.

Claims

1. Method for preparing hybrid biofunctional composites comprising reduced graphene oxide (RGO), polyethylenimine (PEI) and an enzyme, the method comprising the steps of:
   A) providing an aqueous solution of graphene oxide (GO);
   B) reducing the GO by adding PEI to the aqueous solution of GO thereby obtaining an aqueous RGO-PEI solution, and
   C) mixing the aqueous RGO-PEI solution with an enzyme thereby obtaining the hybrid biofunctional composite,

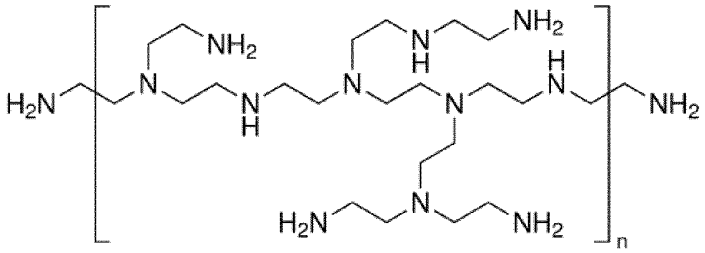
   wherein the RGO and the PEI form covalent bonds and PEI forms a biocompatible matrix electrostatically encapsulating the enzyme inside the matrix.

2. Method for preparing hybrid biofunctional composites according to claim 1, wherein the enzyme has an isoelectric point below 10

3. Method for preparing hybrid biofunctional composites according to any preceding claim, wherein the enzyme is chosen from the group of glucose oxidase (GOx), cholesterol oxidase (ChOx), horseradish peroxidase (HRP), alcohol dehydrogenases (ADH), and Choline oxidase.

4. Method for preparing hybrid biofunctional composites according to any preceding claim, wherein the PEI polymer has an average polymeric length of at least 60.000 monomeric units.

5. Method for preparing hybrid biofunctional composites according to any preceding claim, wherein the PEI polymer has monomeric units with the molecular formula



6. Method for preparing hybrid biofunctional composites according to any preceding claims, wherein after PEI is added in step B), the obtained solution is stirred for between 30 min. – 90 min., or 45 min. – 75 min., or for 60 min. at a **temperature between 70-120 ˚C, or between 80-110 ˚C, or between 90-100 ˚C, or at 95 ˚C.**

7. Method for preparing hybrid biofunctional composites according to any preceding claims, wherein mixing the aqueous RGO-PEI solution with the **enzyme in step C) is done at a temperature between 1-10˚C, or between 2-8˚C, or between 3-6˚C, or between 4-5˚C, or at 4˚C for between 6-24 hours, or** between 8-18 hours, or between 10-14 hours.

8. Method for preparing hybrid biofunctional composites according to claim 6, wherein the mixture obtained in step C) is centrifuged at 8000 rpm for 15 **minutes after being mixed at the temperature between 1-10˚C, or between 2-8˚C, or between 3-6˚C, or between 4-5˚C, or at 4˚C for between 6-24 hours, or** between 8-18 hours, or between 10-14 hours.

9. Method for preparing hybrid biofunctional composites according to any preceding claims further comprising the steps of:
   D) washing the obtained solution in step C) with phosphate buffered saline (PBS), and
   E) successively centrifugating the solution, e.g. three times, to remove loosely bound enzymes.

10. A hybrid biofunctional composite comprising reduced graphene oxide (RGO), polyethylenimine (PEI) and an enzyme, wherein the RGO and PEI form

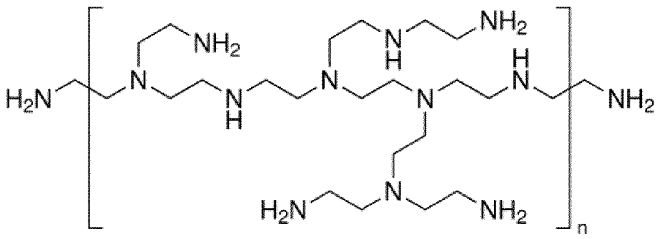
covalent bonds and wherein PEI forms a biocompatible matrix electrostatically encapsulating the enzyme inside the matrix.

11. A hybrid biofunctional composite according to claim 10, wherein the hybrid biofunctional composite is prepared by the method for preparing hybrid biofunctional composites according to any of the claims 1-9.

12. A hybrid biofunctional composite according to any of the claims 10-11, wherein the enzyme has an isoelectric point below 10 and/or is chosen from the group of glucose oxidase (GOx), cholesterol oxidase (ChOx), horseradish peroxidase (HRP), alcohol dehydrogenases (ADH), and Choline oxidase.

13. A hybrid biofunctional composites according to any of the claims 10-12, wherein the PEI has an average polymeric length of at least 60.000 monomeric units.

14. A hybrid biofunctional composites according to any of the claims 10-13, wherein the PEI polymer has monomeric units with the molecular formula



15. Use of the hybrid biofunctional composite according to any of the claims 10-14 as an enzyme-based biosensing material for a graphene based biosensors.

16. Use of the hybrid biofunctional composite according to any of the claims 10-15 for:

- conjugating toxic heavy metal ions such as Pb^{2+}, Hg^{2+} and Cd^{2+};
- clean environmental and water technology; or
- drug delivery where RGO-PEI captures and releases specific drugs.

17. Electrode-composite structures comprising hybrid biofunctional composites according to any of the claims 10-14.

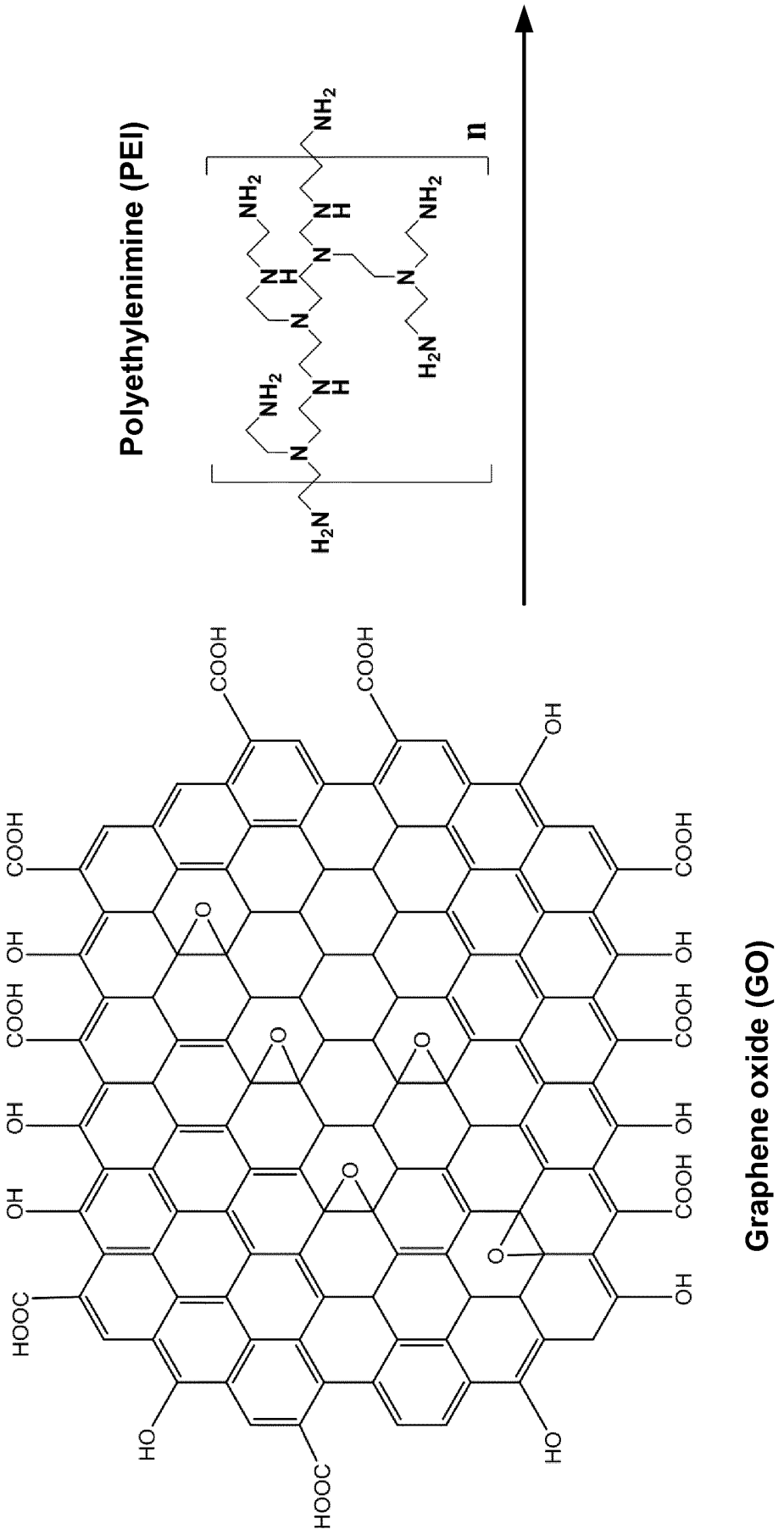

Polyethylenimine (PEI)
Graphene oxide (GO)
COOH
OH
HOOC
HO
O
NH2
H2N
N
H
n

Fig. 1

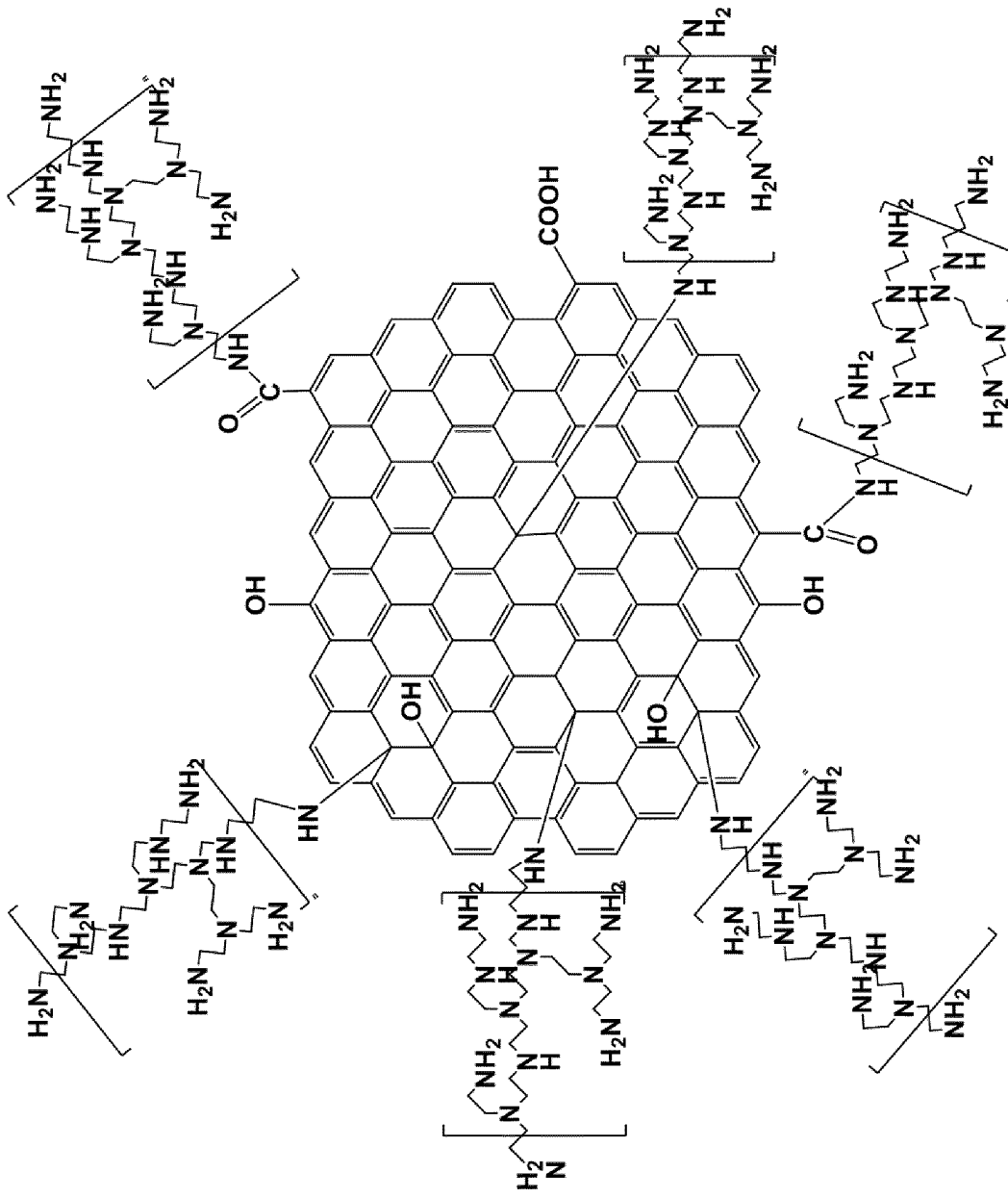


Fig. 1 (continued)

Reduced graphene oxide (RGO)-PEI composite

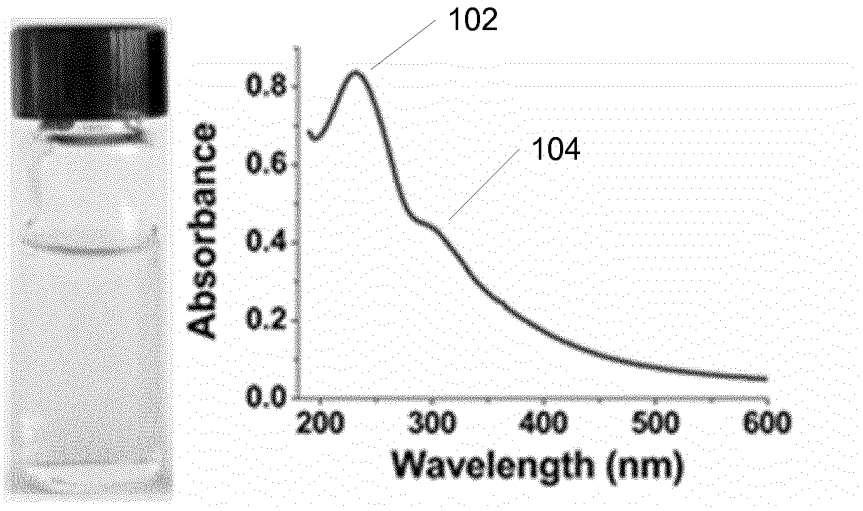


Fig. 2a

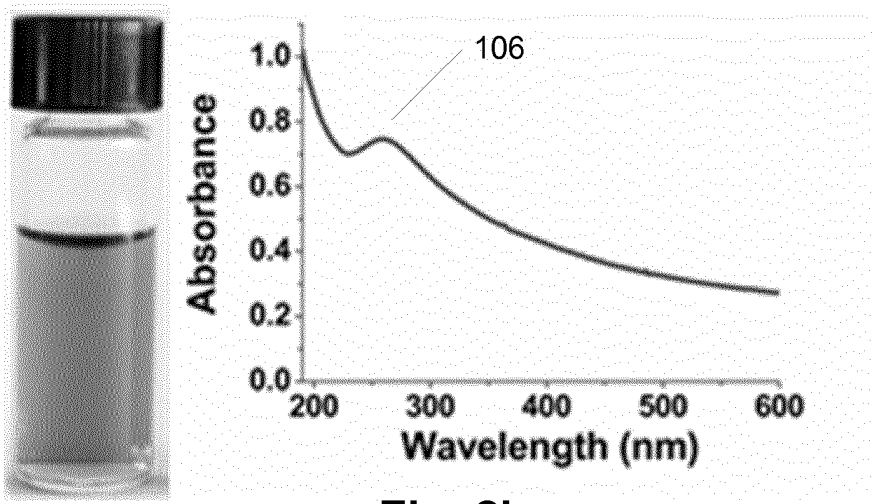


Fig. 2b

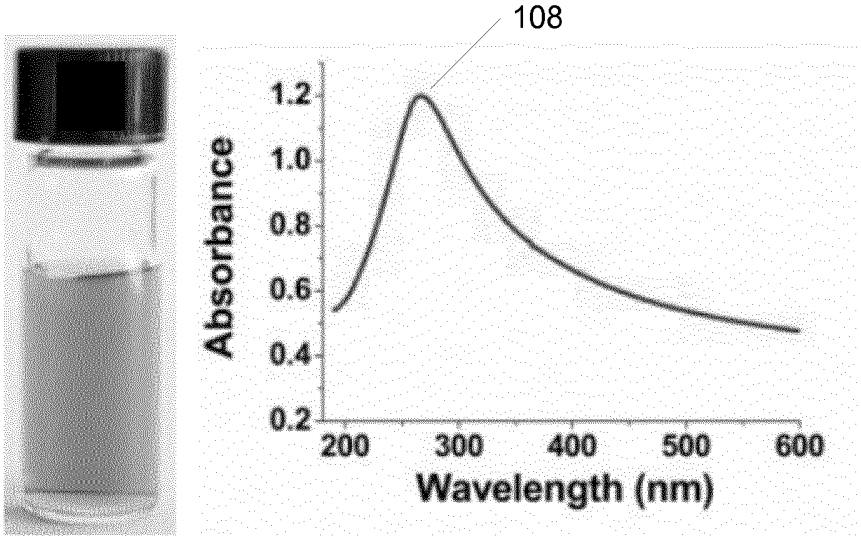


Fig. 2c

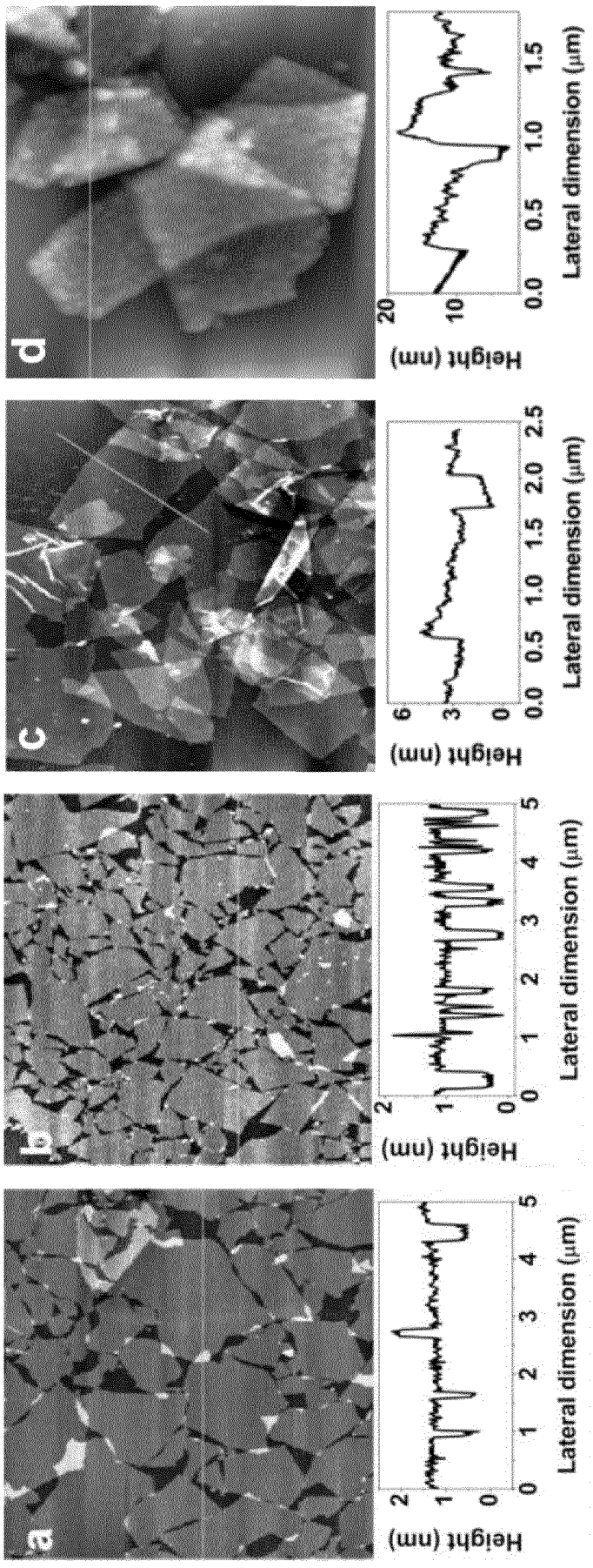

a
b
c
d
Height (nm)
Lateral dimension (μm)
0
1
2
3
4
5
0.0
0.5
1.0
1.5
2.0
2.5
6
3
10
20

Fig. 3

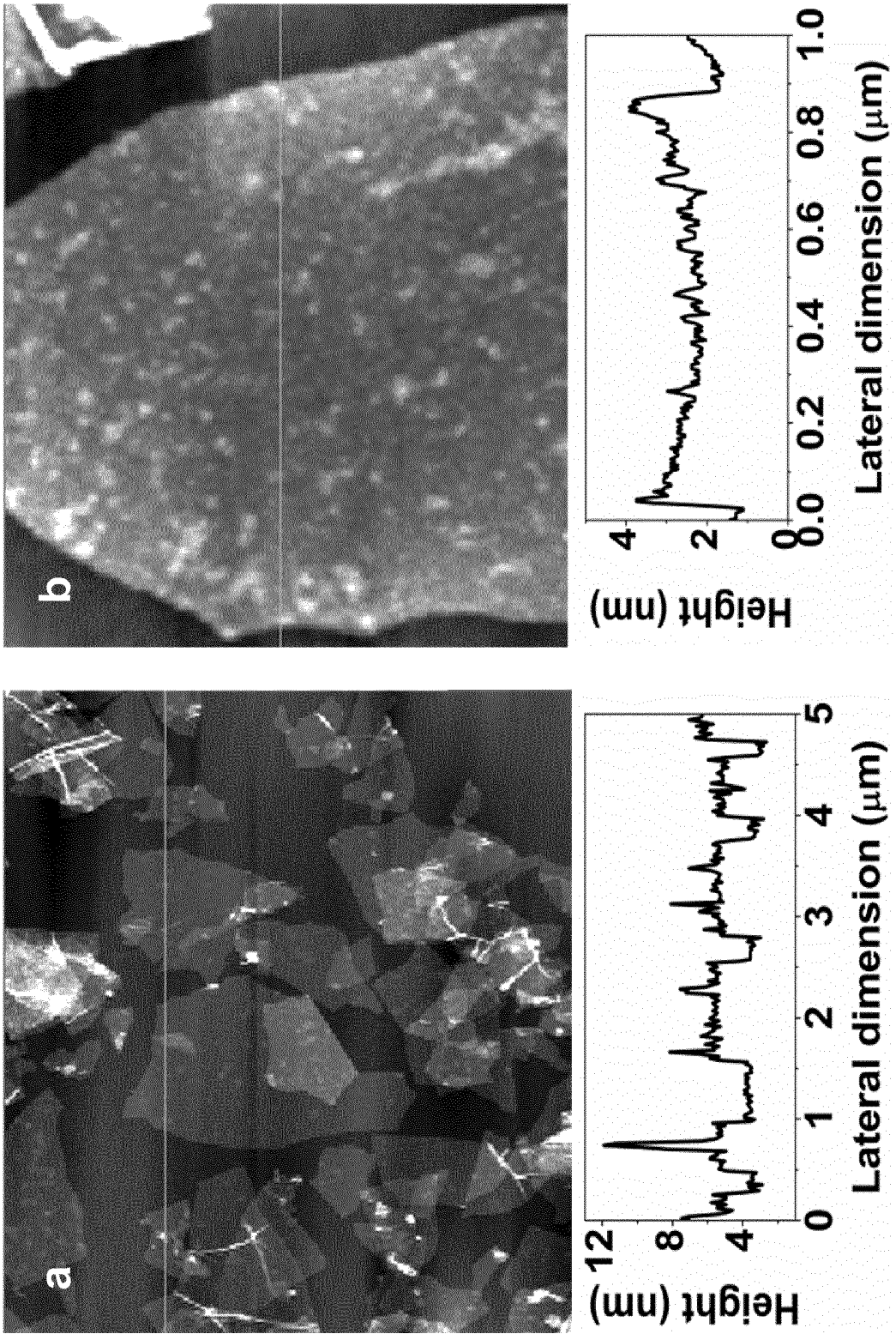

a
b
Height (nm)
Lateral dimension (μm)
0
1
2
3
4
5
4
8
12
Height (nm)
Lateral dimension (μm)
0.0
0.2
0.4
0.6
0.8
1.0
2
4

Fig. 4

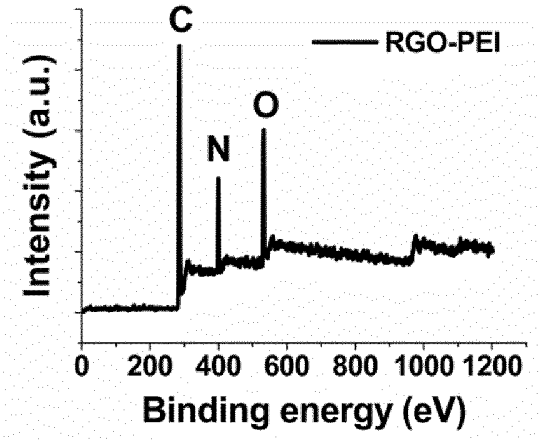


Fig. 5a

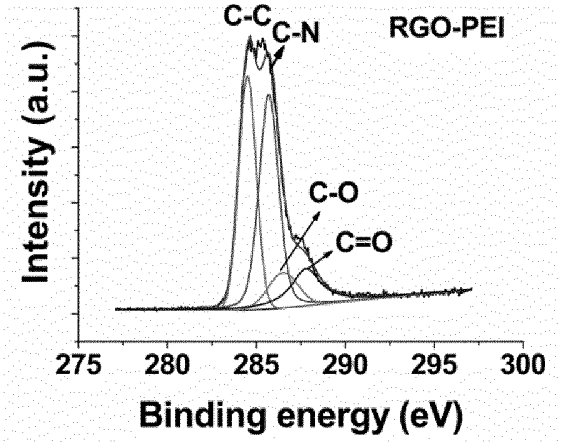


Fig. 5b

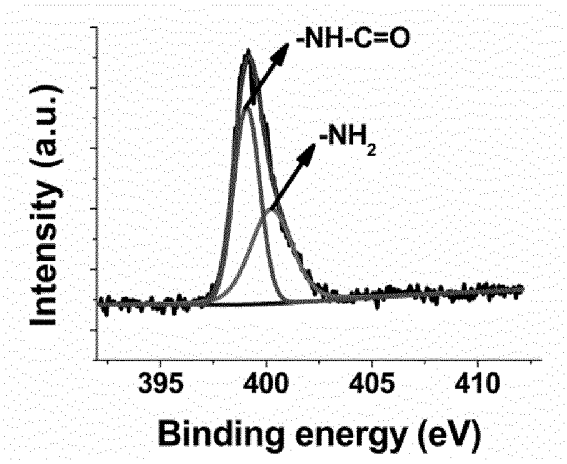


Fig. 5c

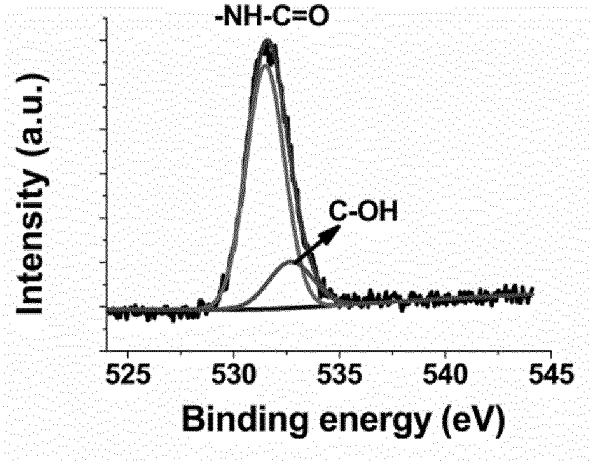


Fig. 5d

	C 1s / %	O 1s / %	N 1s / %
GO	69.46	30.2	0.33
RGO/N_2H_4	85.95	11.64	2.42
RGO/PEI	72	14.44	13.56

Fig. 6

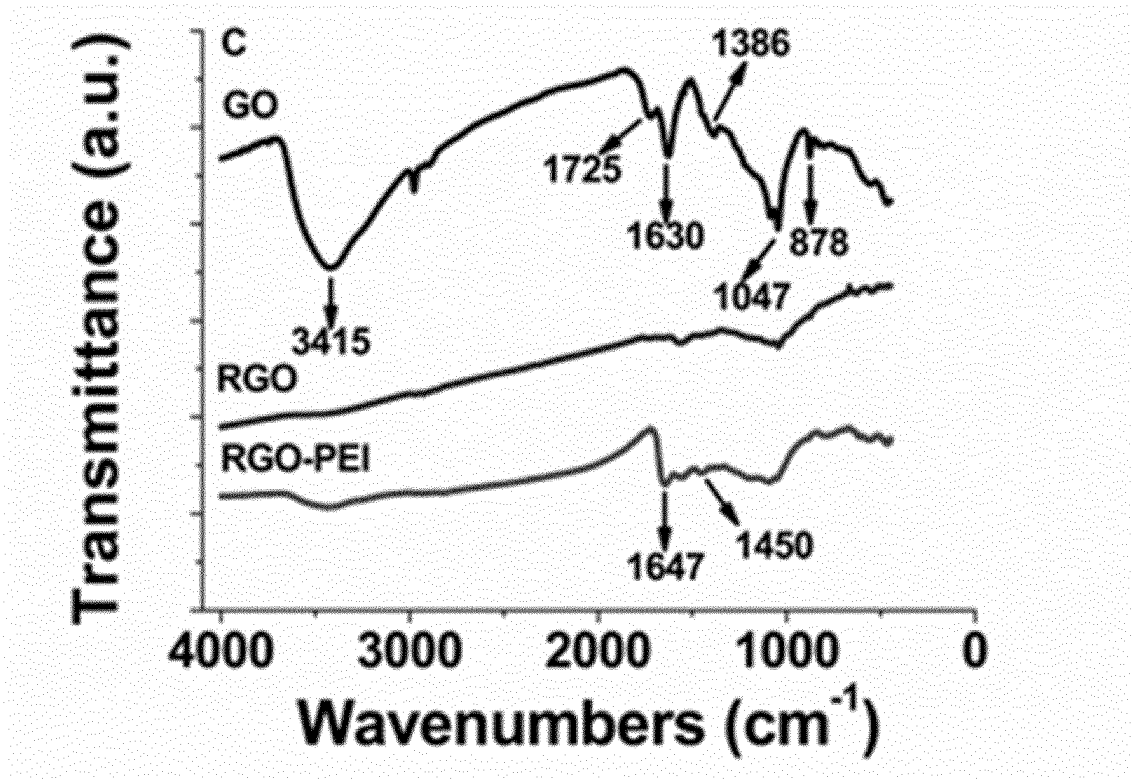


Fig. 7a

	GO	RGO - PEI
O-H	3415	weak
	1386	weak
C=O	1725	1647(N-C=O)
C-O	1047	weak
	878	disappear
Skeletal vibration	1630	
C-N	-	1450

Fig. 7b

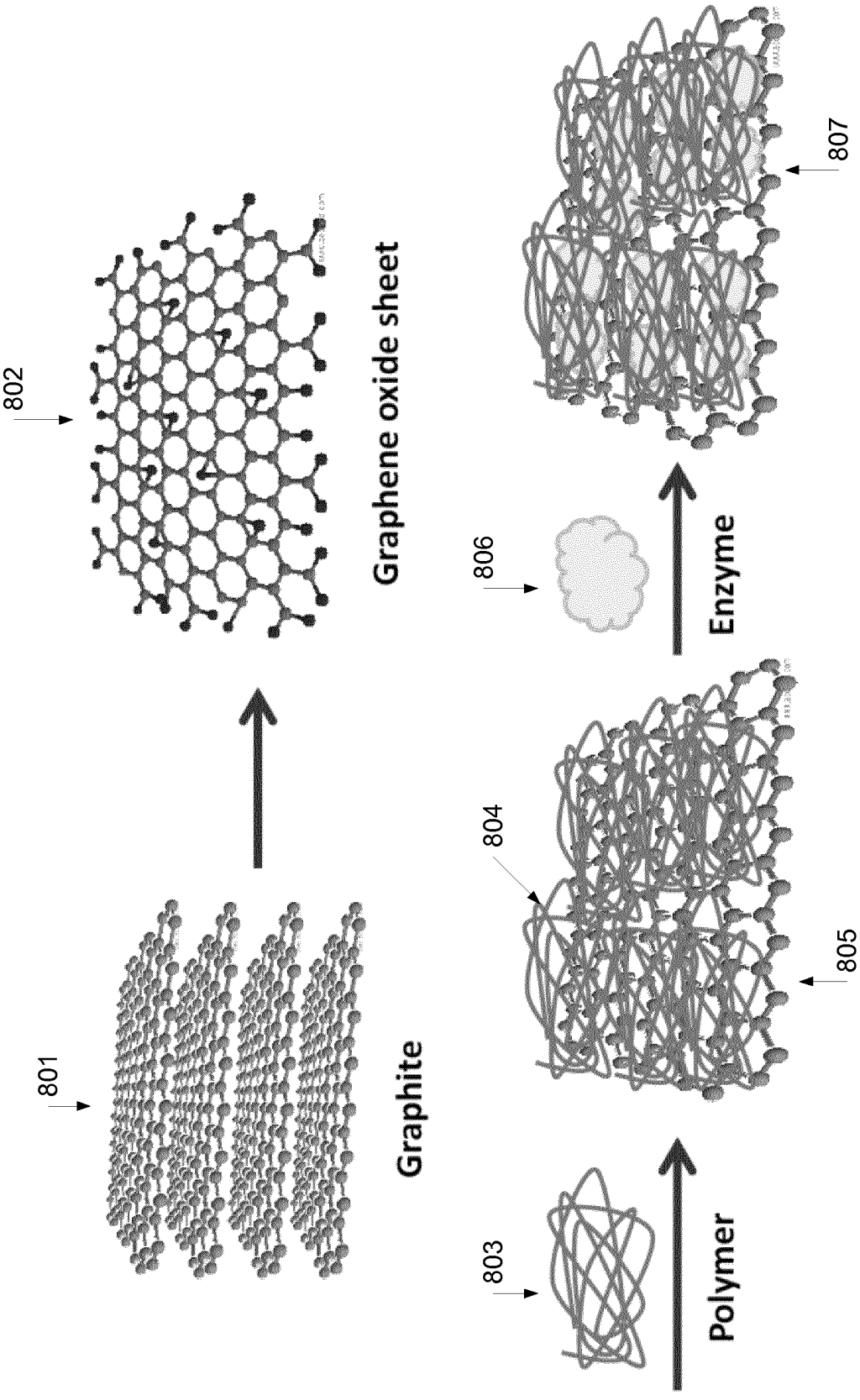

801
Graphite
802
Graphene oxide sheet
803
Polymer
804
805
806
Enzyme
807

Fig. 8

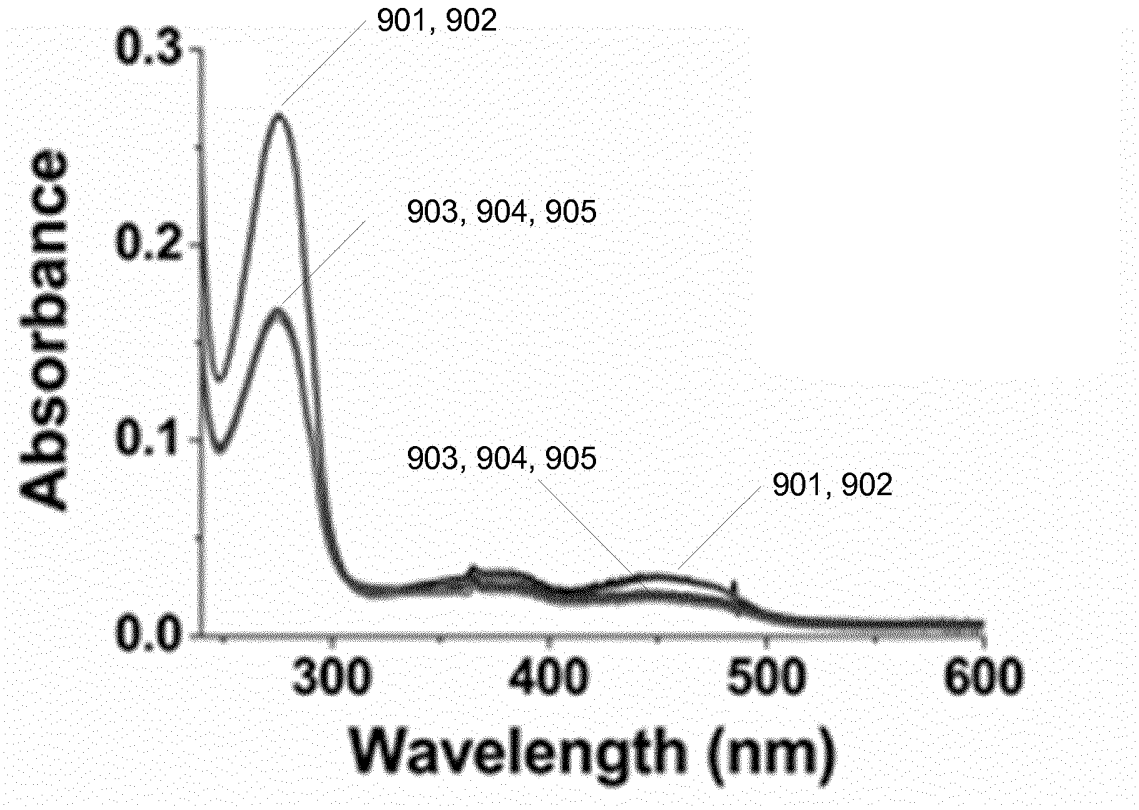

901, 902
903, 904, 905
903, 904, 905
901, 902
Absorbance
0.3
0.2
0.1
0.0
300
400
500
600
Wavelength (nm)

Fig. 9a

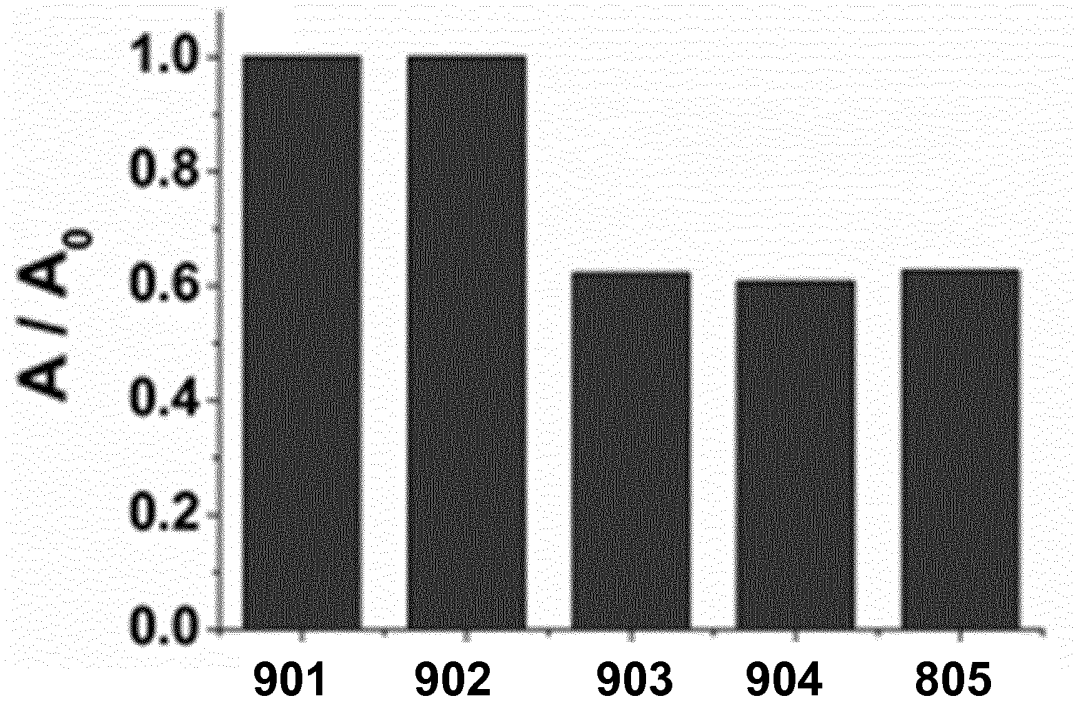

A / A0
1.0
0.8
0.6
0.4
0.2
0.0
901
902
903
904
805

Fig. 9b

Sample	Name	A (277nm)	ΔA	Δm (mg)	m(GOx) / m(RGO-PEI)
901	0.2mg/ml GOx	0.26517	0		
902	0.2mg/ml GOx after centrifugation	0.26510	0.0007		
903	Supernatant of RGO/PEI/GOx after centrifugation	0.16494	0.10023	0.0778	1.95
904		0.16110	0.10407	0.0808	2.02
905		0.16599	0.09918	0.0770	1.93
	Average sample 3-5				1.97

Fig. 9c

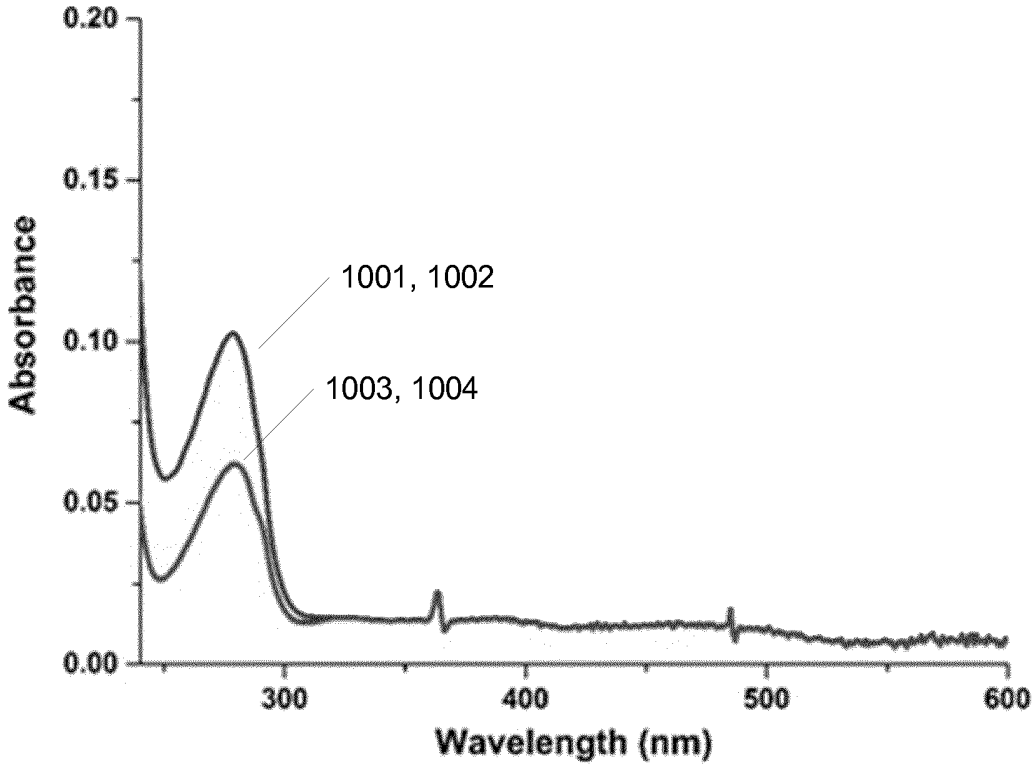


Fig. 10a

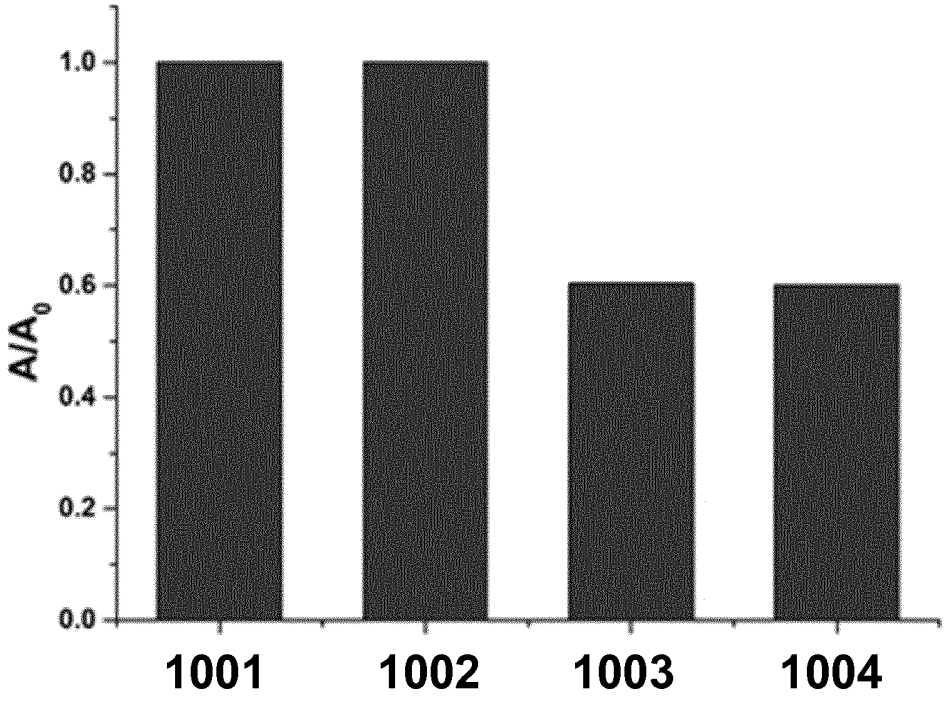


Fig. 10b

Sample	Name	A (277nm)	ΔA	Δm (mg)	m(ChOx) / m(RGO-PEI)
901	0.2mg/ml ChOx	0.1026	0		
902	0.2mg/ml ChOx after centrifugation	0.10262	0.00002		
903	Supernatant of RGO/PEI/ChOx after centrifugation	0.06192	0.04068	0.0793	1.9825
904		0.0616	0.041	0.08	2.0
	Average sample 3-4				1.9912

Fig. 10c

Fig. 11a

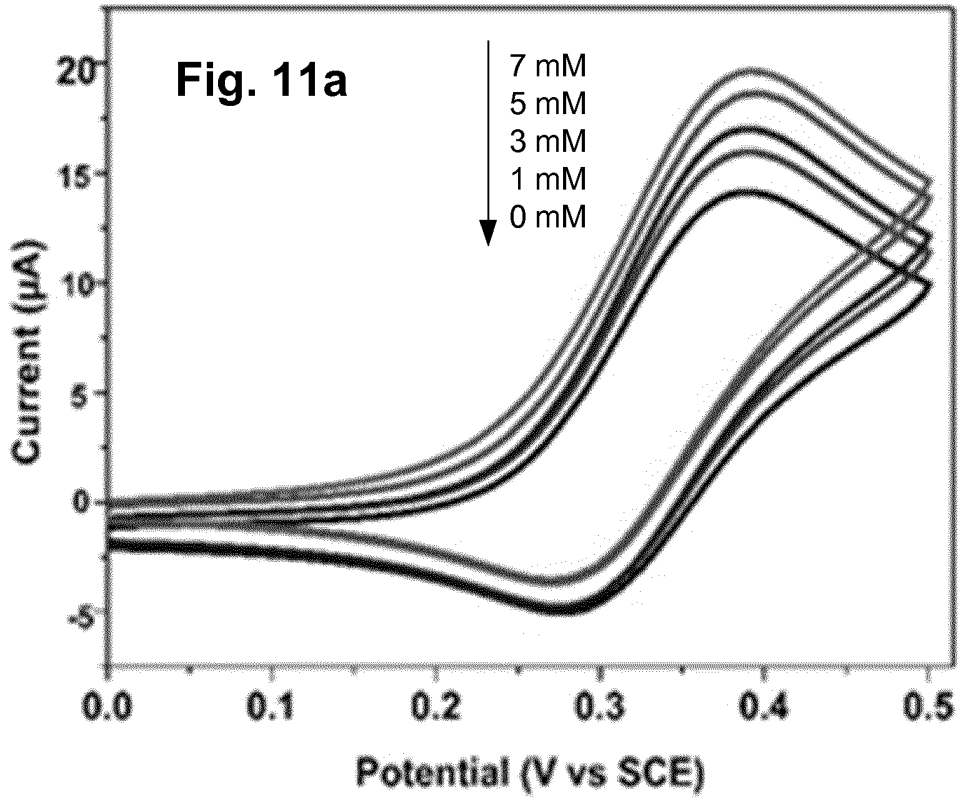


Fig. 11b

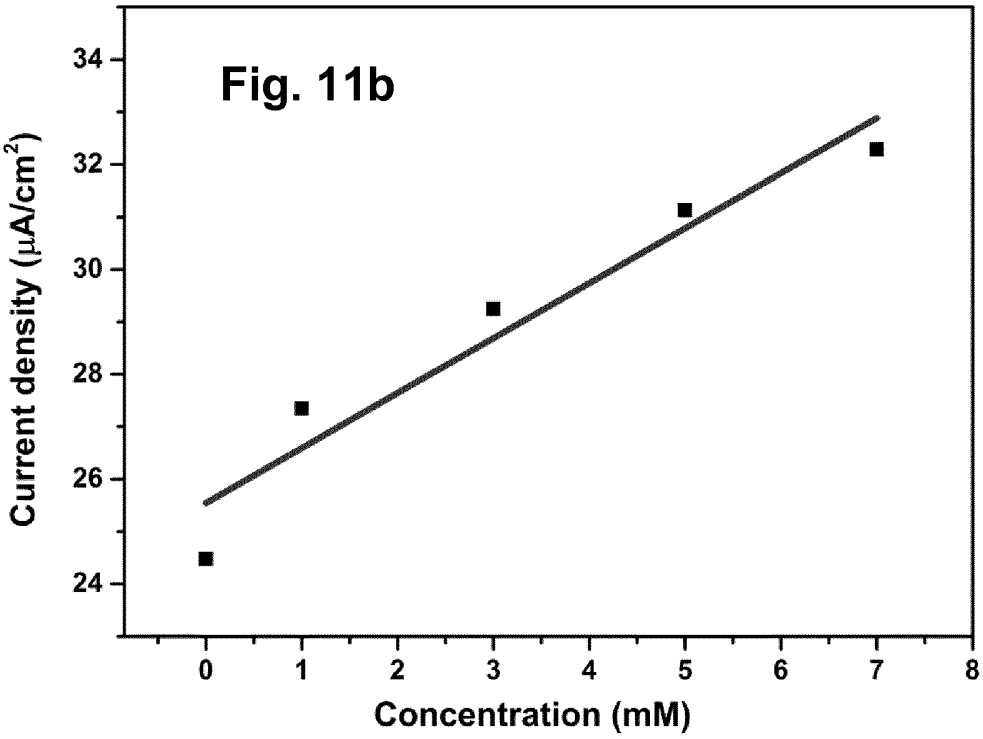


Fig. 12a

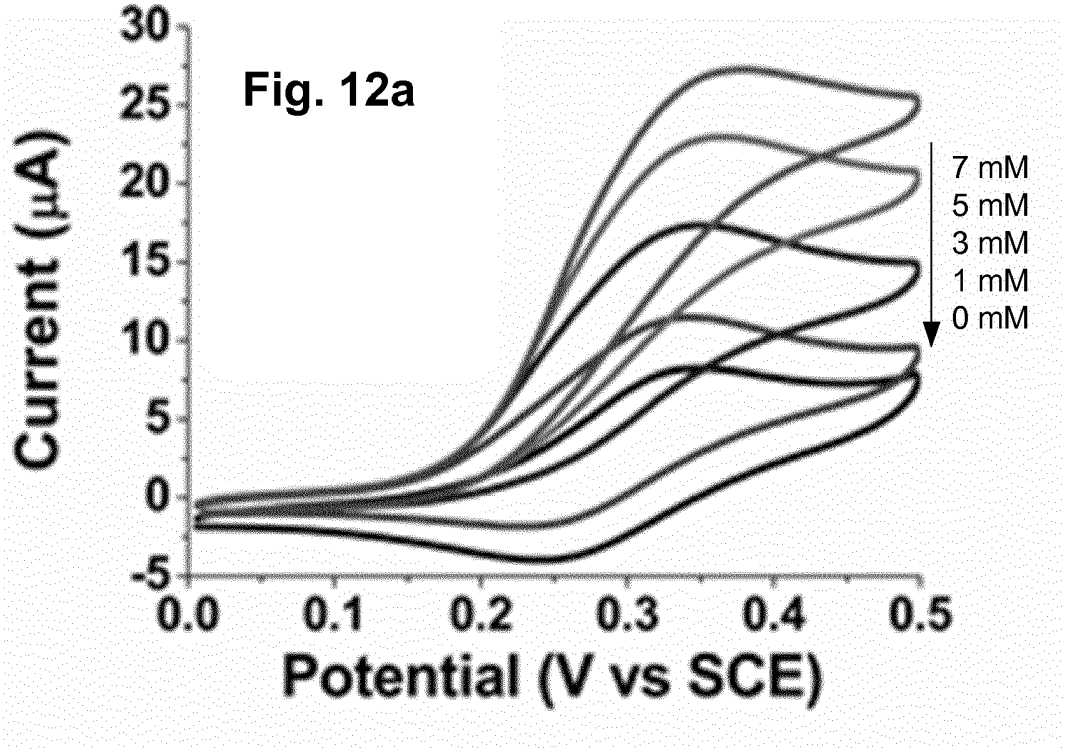


Fig. 12b

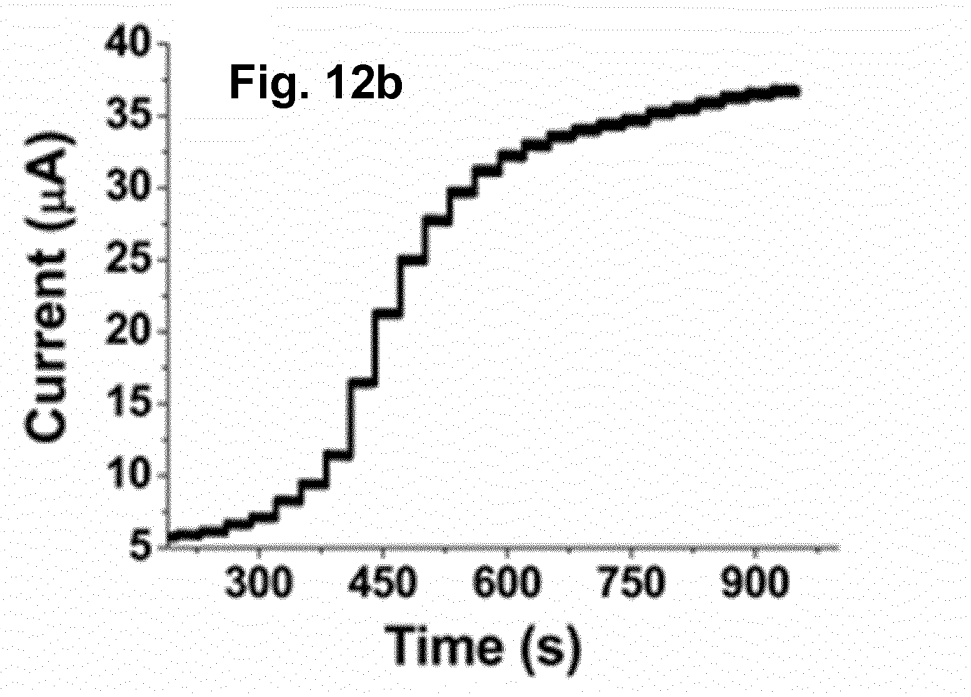


Fig. 12c

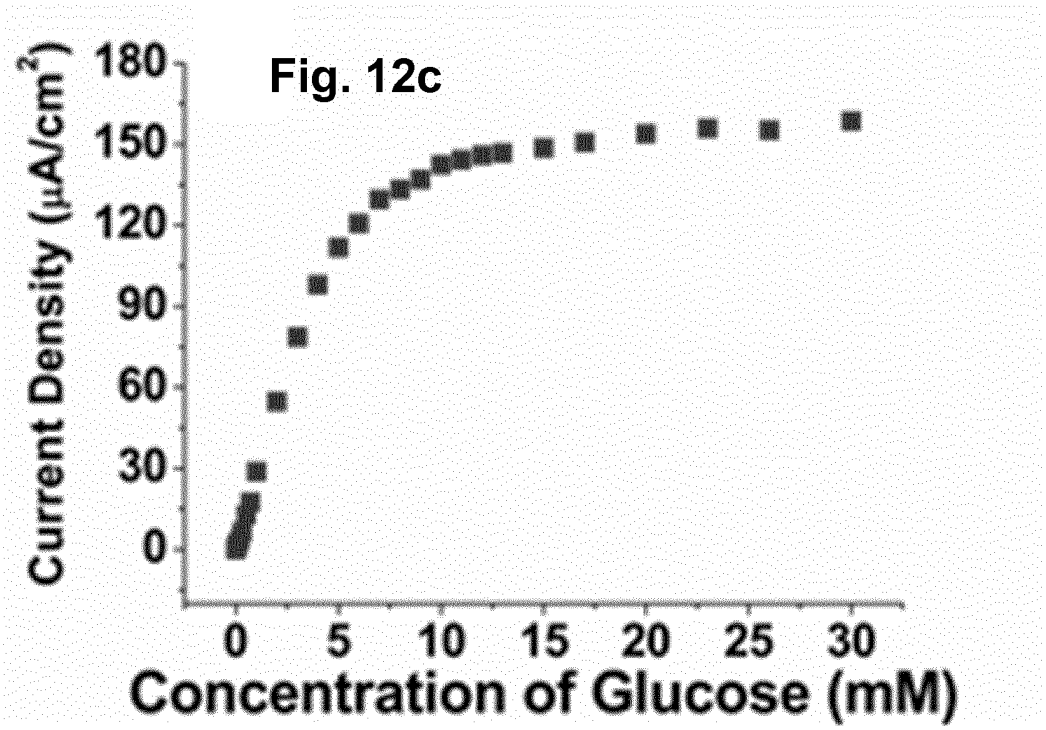
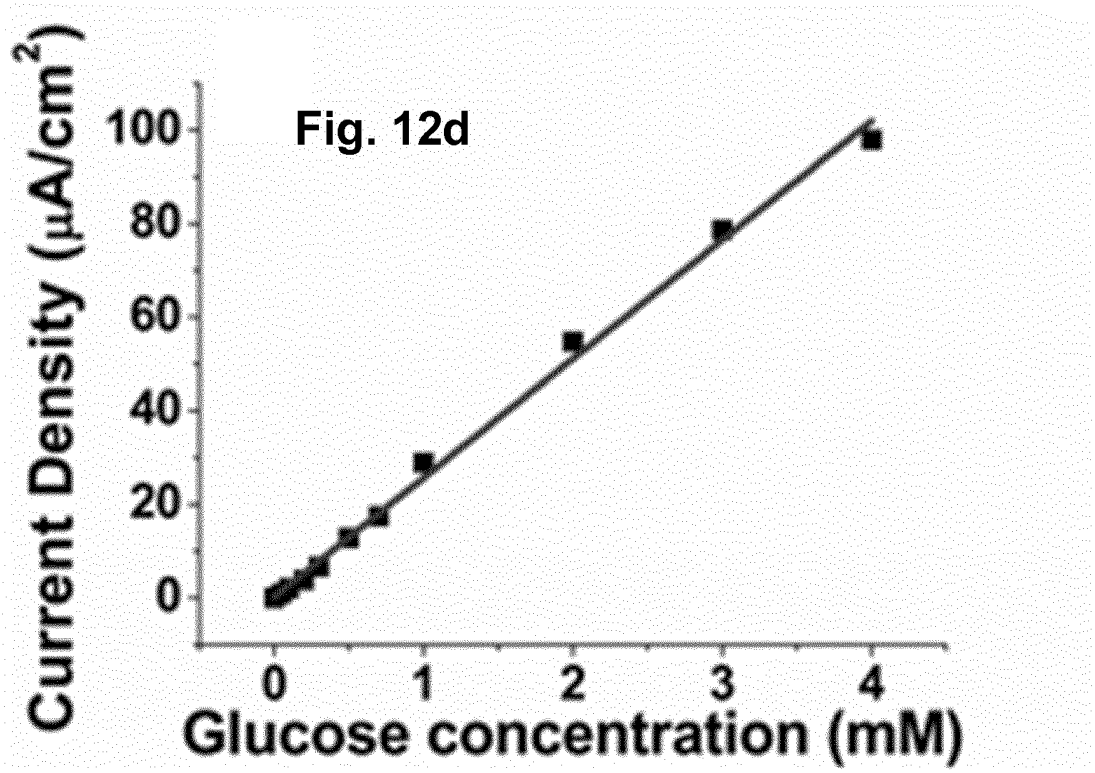


Fig. 12d



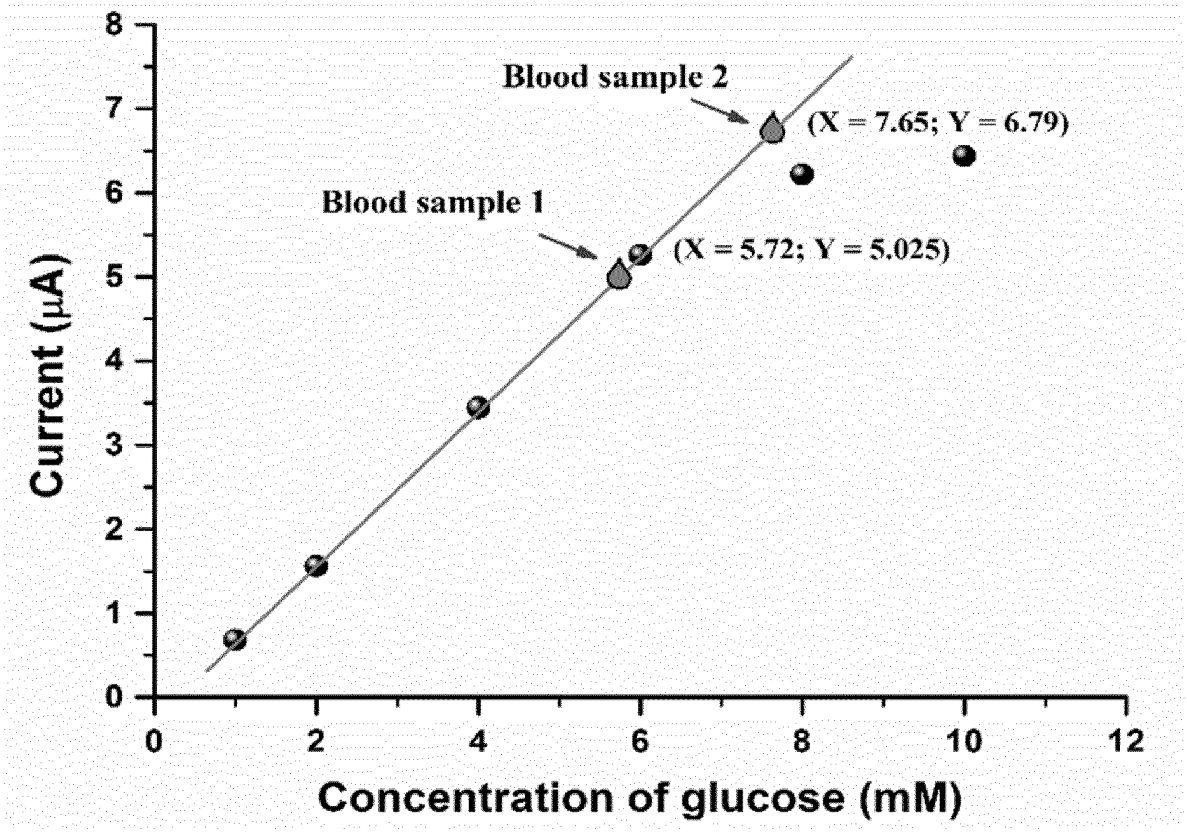


Fig. 13a

	Concentration of blood (mM)	
Blood sample	Biosensor of the invention	Commercially available blood glucose monitoring device
Sample 1	5.72	5.2
Sample 2	7.65	7.3

Fig. 13b

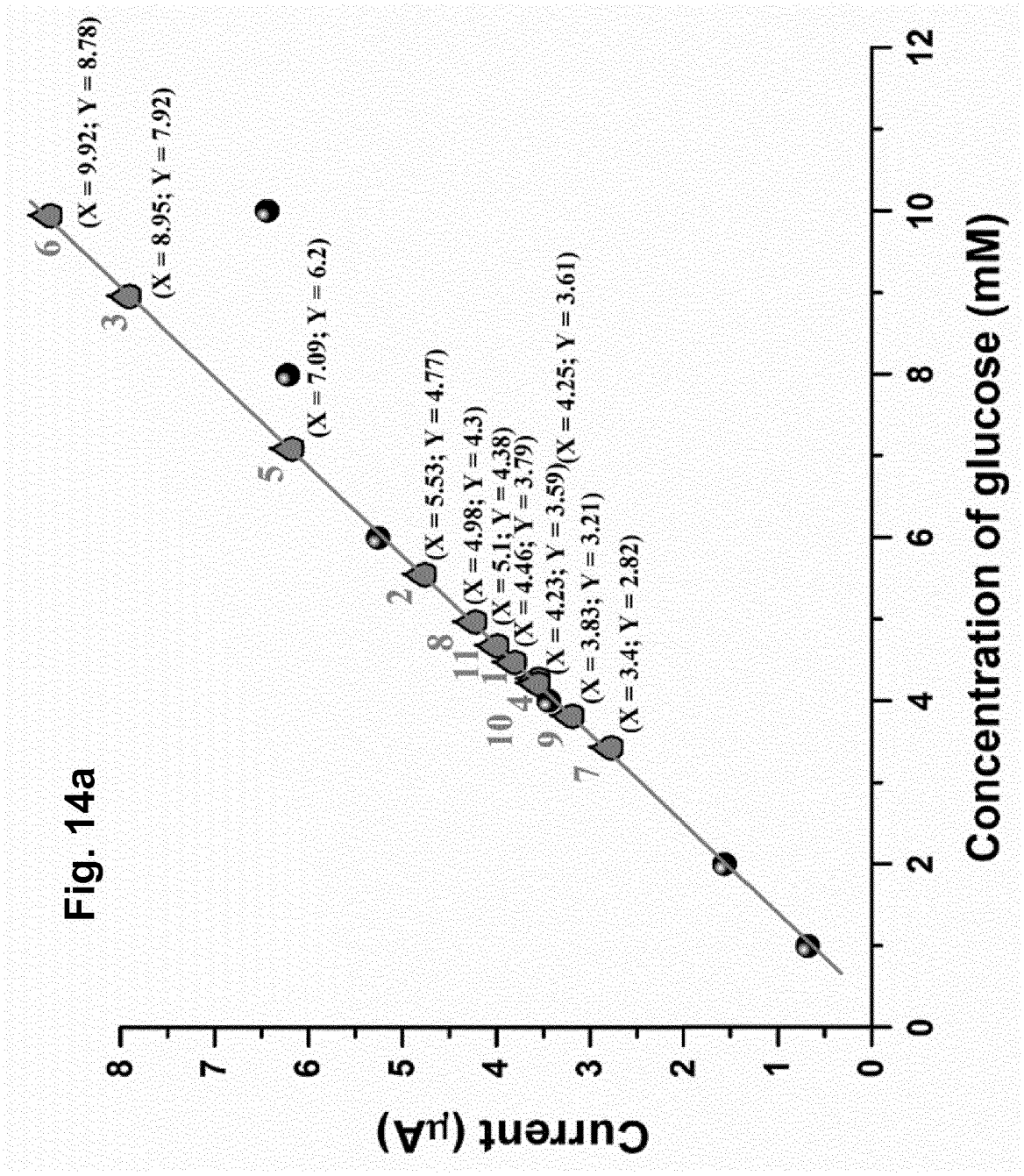
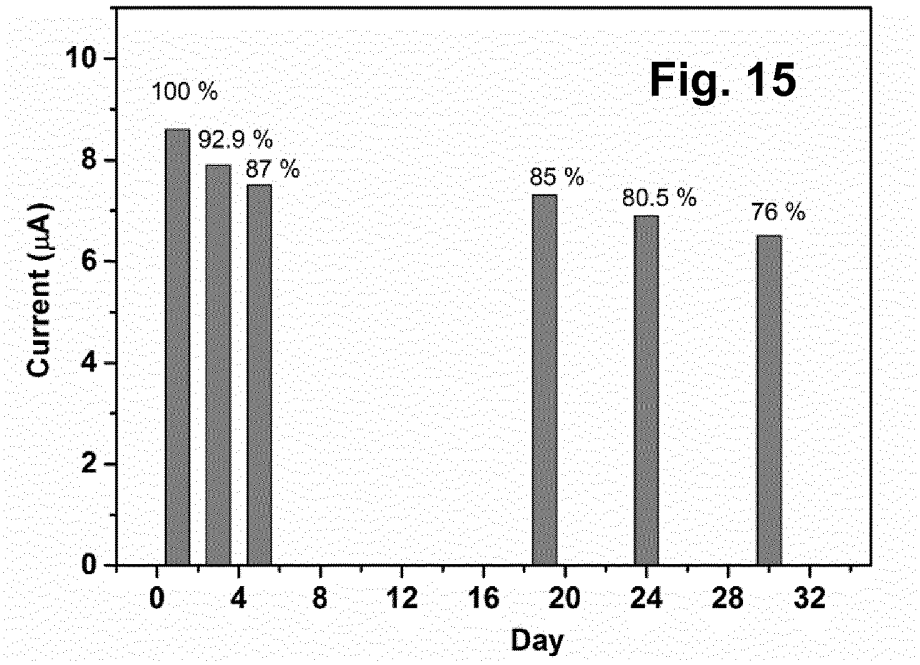


Fig. 14a
Current (μA)
Concentration of glucose (mM)
0
1
2
3
4
5
6
7
8
0
2
4
6
8
10
12
(X = 9.92; Y = 8.78)
(X = 8.95; Y = 7.92)
(X = 7.09; Y = 6.2)
(X = 5.53; Y = 4.77)
(X = 4.98; Y = 4.3)
(X = 5.1; Y = 4.38)
(X = 4.46; Y = 3.79)
(X = 4.23; Y = 3.59)
(X = 4.25; Y = 3.61)
(X = 3.83; Y = 3.21)
(X = 3.4; Y = 2.82)
6
3
5
2
8
11
1
4
10
9
7

	Concentration of blood (mM)	
Blood sample, Glostrup Hospital	Biosensor of the invention	Commercially available blood glucose monitoring device
Sample 1	4.46	4.9
Sample 2	5.53	5.8
Sample 3	8.95	9.4
Sample 4	4.23	4.4
Sample 5	7.09	7.2
Sample 6	9.92	10.8
Sample 7	5.4	6.2
Sample 8	4.98	5.3
Sample 9	3.83	4.1
Sample 10	4.23	4.8
Sample 11	5.10	5.4

Fig. 14b



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/076933

A. CLASSIFICATION OF SUBJECT MATTER
INV. B82Y30/00 G01N27/327
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
B82Y G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, CHEM ABS Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 102 850 795 B (UNIV ZHEJIANG SCIENCE & TECH) 16 April 2014 (2014-04-16) the whole document	1-3, 10-12, 15-17
Y	HONGYU LIU ET AL: "In situ synthesis of the reduced graphene oxide-polyethyleneimine composite and its gas barrier properties", JOURNAL OF MATERIALS CHEMISTRY A, vol. 1, no. 11, 1 January 2013 (2013-01-01), page 3739, XP055179493, ISSN: 2050-7488, DOI: 10.1039/c3ta01228j the whole document	1-17

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
14 January 2016	28/01/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Pinheiro Vieira, E

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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